United States Environmental Protection Agency

LABORATORY ANIMAL PROJECT REVIEW

Please note:

1. All information in this LAPR is considered privileged and confidential by the IACUC and regulatory authorities.
2. Approved LAPRs are subject to release to the public under the Freedom of Information Act (FOIA). Do not include proprietary or classified information in the LAPR.
3. An approved LAPR is valid for three years.

LAPR Information

LAPR Title:	Testing predicted chemical reactivity-based neurotoxicity to develop possible Adverse Outcome Pathways (AOPs) in rats
LAPR Number:	20-01-002
Principal Investigator	Exemption 6
Author of this Document:	Exemption 6 RTP/USEPA/US
Date Originated:	10/25/2016
LAPR Expiration Date:	01/31/2020
Agenda Date:	01/25/2017
Date Approved:	01/26/17
Date Closed:	

APPROVALS

APPROVER	NAME	APPROVAL DATE	COMMENTS	
	Exemption 6 Exemption 6 Exemption 6 RTP/USEPA/US by Exemption 6 /RTP/USEPA/US	01/25/2017		
	Exemption 6 /RTP/USEPA/US by Exemption 6 /RTP/USEPA/US	01/26/2017		
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Administrative Information

1. Project Title (no abbreviations, include species):
Testing predicted chemical reactivity-based neurotoxicity to develop possible Adverse Outcome Pathways (AOPs) in rats

Is this a continuing study with a previously approved LAPR?
No

2. Programatic Information
a. What Program does this LAPR support? Please provide the Research Program, Project, Task Number and Title.
Program: CSS
Project: Adverse Outcome Pathway Discovery and Development (AOPDD)
Task: 17.01.1.1F - Linking predictable chemical reactivity-based protein adduct formation to a variety of target organ toxicities – development of putative AOPs
b. What is the Quality Assurance Project Plan (QAPP) covering this project?
E-TAD-0030301-2017-01

3. EPA Principal Investigator/Responsible Employee:

Principal Investigator	***Phone Number***	***Division***	***Mail Drop***
Exemption 6	Exemption 6	TAD	MD
	Lotus Notes Address	***Branch***	B105-04
	Exemption 6 Exemption 6 Exemption 6 Exemption Exemption RTP/USEPA/US	NB	

4. Alternate Contact:

Alternate Contact	***Phone Number***	***Division***	***Mail Drop***
Exemption 6	Exemption 6	TAD	MD
	Lotus Notes Address	***Branch***	B105-04
	Exemption 6 Exemption 6 Exemption Exemption Exemption RTP/USEPA/US	NB	

SECTION A - Description of Project

1. Explain the study objective(s) in non-technical language such that it is understandable by non-scientific persons. Explain how the benefits from the knowledge gained from this research outweigh the costs to the animals used in this research. If this is a continuing study from a previous LAPR, briefly justify the continuation. Please spell out all acronyms and abbreviations with their initial use.

Multiple environmental chemicals and disease states have been shown to produce peripheral neuropathies in humans. Peripheral neuropathy occurs when the nerves of the peripheral nervous system are impaired. This form of toxicity often results in tingling or numbness of the hands or feet, with possible muscle weakness. Some chemical-induced neurotoxicity has been shown to be caused by the chemicals irreversibly binding to neuronal proteins and destroying the functionality of the protein. The ability of certain classes of chemicals to bind stably to proteins has been modeled based on their chemical reactivity.

We have used such chemical modeling on additional classes of chemicals to predict their potential to bind to proteins and produce neurotoxicity. Chemicals were selected from ToxCast list and the Office of Water's Contaminant Candidate List (OW CCL). They were screened for possible protein binding using structure-activity relationship analysis, then ranked for potential to bind to proteins using chemical reactivity modeling.

We propose to use two chemicals (that were computationally predicted to produce protein binding). The first is citronellal, which is found in edible and medicinal plants and is a major component of the essential oil of such plants. Exposure can occur through food or water sources (on the OW CCL) and is included in the list of Toxcast chemicals. The second chemical is phenylacetaledhyde, which is found in cosmetic, personal care, chemical, and pharmaceutical agents. It has been found to be a moderately strong sensitizer for allergic contact dermatitis using in vitro assays. Exposure can be oral, and this chemical is included on the OW CCL and Toxcast chemical list. We are not aware of epidemiological studies linking these chemicals to effects in humans, but citronellal has been shown to have antinociceptive (pain reducing) effects in rodent models.

The purpose of these studies is to test these computational toxicity predictions in an in vivo model to see if the chemicals produce neurotoxicity with repeated exposures (required for bound proteins to accumulate). The chemicals will be selected from structural classes not used in developing the chemical reactivity models. If successful, this work will provide some confidence in the models ability to predict neurotoxicity. If the toxicity predictions are verified, this work will provide support for using the computer models to screen thousands of untested chemicals to prioritize for further in vivo testing, thereby reducing the use animals for testing low priority chemicals.

We will use functional behavioral assessments, nerve excitability testing (Compound Muscle Action Potentials [CMAPs], Sensory Nerve Action Potentials [SNAPs]), and compound nerve action potentials (CNAP; the size of the nerve's signal) and nerve conduction velocity (NCV; the speed that a nerve moves the signal along its body) to quantify neurotoxicity. We will also use somatosensory evoked potentials (SEPs) to assess nerve function in the central nervous system. Our laboratory has extensive experience with all of the proposed test methods.

2. Scientific rationale for proposed animal use.

a. Why is the use of animals necessary?

While nerve excitability testing is used clinically in humans, and has been adapted to rodents, it can only be studied in intact animals. Testing SEPs/CNAPs/NCV also requires intact functioning nerves. There are no good in vitro models of peripheral neuropathy. The goal of this work is to test predictive computer models in animal models. This is required to build scientific confidence in the computer model predictions.

b. Justify the species requested:

The rat has been used extensively as a model in physiological toxicology because of extensive knowledge of its anatomy, the historical database of neurophysiological alterations after treatment with different agents, its size, and the general use of rats in toxicological testing. Furthermore, the rat model of neurological function is a commonly used animal model to predict human neural dysfunction. Additionally, much of the data submitted to the EPA for chemical registration is collected in rodents. Use of the same animal model will increase the ability to interpret the data collected by different laboratories, and maintain consistency with the data previously collected in our laboratory.

3. How was it determined that this study is not unnecessary duplication?

A literature search has determined that there are no studies that document the use of nerve excitability testing, CNAPs, or NCV to examine peripheral neurotoxicity produced by the proposed chemicals in rodents. We used PubMed with the key words: Nerve excitability, nerve action potential, nerve conduction velocity, rodents, and the

proposed toxicant name (see attached).

SECTION B - In Vivo Procedures

1. Briefly describe the experimental design. Include descriptions of the age, weight and sex of the animals. Supplementary information may be attached at the end of the LAPR, but please include critical information within the body of the LAPR.

Male Long Evans rats of approximately 90 - 120 days old at the time of neurophysiological testing will be used.

Each chemical will be tested in a two step process. The first phase will determine if overt toxicity is observed over a 2 week period. Signs of non-transient pain, distress, or hindlimb paralysis will be monitored. Signs of pain and distress will include lethargy and decreased muscle tone, mild tremors, raspy breathing, or repeated and prolonged vocalizations not associated with handling and testing. Overall changes in health status such as a rough, ungroomed coat, or weight loss (as opposed to less weight gain) will also be monitored. During weighing the animals, they will have cage side observations for changes in gait, and assessment of foot splay during the weighing process. If overt toxicity is observed, treatment will be stopped and the animals euthanized. This first phase will be used to determine the range of doses in the second study.

In the second phase, animals will be dosed with vehicle or test compound for 30 days. If no effects are observed in the behavioral testing, treatment will continue for an additional 30 days (up to 60 days total).

We will use citronellal and phenylacetaledhyde as the first two chemicals to be tested.

Time Line:

Assuming the treatment duration is for 4-5 weeks:

1. During weeks 1-3, selected functional assessments (e.g. gait score, foot splay, grip strength) will be performed weekly during treatment. These are measures in rats that can be used to understand how well people can walk or grip objects, and may imply abnormal function of the nervous or muscular systems.
2. During week 4, nerve excitability testing procedures will be performed on the same animals.
3. During week 5, the same animals will be implanted with epidural cranial electrodes to allow assessment of nerve function in the central nervous system (SEPs).
4. During week 6, the same animals would be tested for SEPs/CNAPs to assess central nerve function and a second measure of peripheral nerve function.

Notes:

1) Dose range studies are listed as a Category C procedure.
2) The SEP/CNAP procedure is listed as a Category E procedure based on restraint.

2. Justify the number of animals. Include explanation (e.g., biological, statistical, regulatory rationale) for the number of animals needed for each treatment group, and the overall number requested for the duration of the LAPR.

This LAPR includes testing 2 chemicals. Additional chemicals may be added at future dates.

This research will proceed in two steps:

Step 1. Each chemical will be tested at 4-5 concentrations with 3-4 rats/concentration for two weeks. This step will require a maximum of 20 animals/chemical.

Step 2. Each chemical will be tested at 4 concentrations (control + three levels of test compound) using 20 rats per concentration. Previous experience with these tests have shown that about 15-18 animals are required for statistically reliable results. The additional animals will allow for the possibility of bad electrodes, surgery, etc... This step will require 80 animals/chemical.

3. State how many animals over the study period are expected to be used under the following three categories of pain/distress (USDA nomenclature as defined in the instructions): Please enter numbers only.

Categories	Adults	Offspring
C) Minimal, transient, or no pain/distress:	40	
D) Potential pain/distress relieved by appropriate measures:		
E) Unrelieved pain/distress:	160	

4. Does this LAPR include any of the following:

☒ Restraint (>15 Minutes) ☒ Survival surgery

☐ Food and/or water restriction (>6 Hours) ☐ Non-survival surgery

a. Please provide a scientific justification. Describe how animals will be monitored, how health status will be tracked, and what records will be maintained.

The brain's responses vary over the different regions of the cortex. Thus, to maintain constant recording locations between animals (thus reducing experimental variance and the number of animals required) stereotaxic surgery will be used. This implantation will assure that the electrodes will not move during the testing procedure, which must be performed in awake animals.

During surgery, animals are maintained on a recirculating water heating pad, and are monitored both with visual examination and a pulse oximeter. Following surgery, the animals are monitored in their home cages for any signs of infection Exemption 6 If any signs of infection are observed, the attending veterinarian (AV) will be contacted for treatment options.

Restraint is required to assure consistent location of the animal relative to the somatosensory stimuli. Differences in location of an animal relative to the stimulus results in different physiological responses, increasing variability of the measured endpoints, therefore requiring additional animals for proper statistical design.

Animals will be habituated to the restraint procedure in their home cage for 2 days prior to testing to reduce distress (5 min on 1st day, 15 min on 2nd day). Animals will be tested on day 3. Immediately after each restraint session, the animal will be rewarded with a treat (e.g., sugar pellet, fruit crunchies, vanilla wafer). During the habituation days, the animals will be observed for excessive struggling. They will be removed from the restraint if they show signs of respiratory distress. Monitoring will be performed by Exemption 6

The IACUC (including the AV), has viewed this procedure in person, including the restraint with tape and the electrodes that will be used in this testing LAPR. The procedure was categorized as Category E due to restraint. The IACUC (including the AV), watched the recovery period of the animal after removal from the restraint, which was observed to be short and smooth, and then animals are returned to home cages. We keep detailed written records of anesthesia, analgesia, and recovery. These records are maintained on the animal holding room door while the animals are being dosed with analgesics. When they are done with these doses, records are maintained in the lab.

5. Category C procedures. Describe each procedure separately, include details on the following:

a. Treatments (e.g., dosages, duration of exposure, route, volume, frequency):

As described elsewhere, chemicals will be tested in a 2 step process. In each step, the chemical will be dissolved in food grade corn oil.

Step 1 is a dose ranging study that will last for 14 days using 4-5 concentrations of chemical

The first chemical to be tested will be citronellal. Because of the lack of data (see Section 9), Step 1 dosing concentrations will be based on proportions of the LD50. Citronellal (LD50 Rat oral = 2420 mg/kg) will be diluted in corn oil and administered via oral gavage. Pilot studies will be used to establish dose ranges. We will start with LD50 * 0.1 = 242 mg/kg/day and adjust concentrations based on what is observed in the animals.

The second chemical to be tested will be phenylacetaledhyde. Because of the lack of data (see Section 9), Step 1 dosing concentrations will be base on proportions of the LD50. Phenylacetaldehyde (LD50 Rat oral = 1,550 mg/kg) will be diluted in corn oil and administered via oral gavage. Pilot studies will be used to establish dose ranges. We will start with LD50 * 0.1 = 155 mg/kg/day and adjust concentrations based on what is observed in the animals.

Step 2 is the testing phase that will last for up to 60 days using 3 concentrations of chemical plus a vehicle control group. The concentrations in Step 2 will be based on the results of Step 1. We will attempt to use similar percents of the LD50 for each chemical.

b. Survival Blood Collections (method, volume, frequency):

N/A

c. Testing methods (including non-stressful dietary restrictions/modifications, mild non-damaging electric shock):

Step 2: Chemical Testing

A. Behavioral Testing

Behavioral testing will occur weekly during dosing before nerve excitability testing. Male Long Evans rats will be evaluated for changes in health and general appearance. Subjective evaluation will be made while the rat is freely moving on a laboratory cart, with the observer ranking the appearance and degree of gait abnormality or ataxia as well other notable signs (e.g., altered arousal). Forelimb and hind limb grip strength (having the rat pull on a wire mesh screen attached to a strain gauge) and landing foot splay (measuring the distance between paws when landing from a drop 30 cm high) will be quantified. These tests are components of the functional observational battery (FOB) that is regularly used at EPA, and the scientific staff has extensive experience with the test methodology.

See Section B.6 for mild electrical stimuli used to stimulate peripheral nerves.

d. Animal restraint and confinement beyond routine housing and handling. Include a description of the type of restraint device, acclimation to device, duration of restraint:

See Section B.6.c.

e. Breeding for experimental purposes (e.g. length of pairing, number of generations):

N/A

f. Describe how animals will be identified and monitored. Include description of identification procedures. (For example, if transponders are used, how are the animals prepared?) Include frequency of observations and by whom:

Animals will be identified on cage cards. Tail marking with ink pens may be used prior to testing.

Chemical dosing: Animals will be monitored 5 days/week by Exemption 6 or other TAD staff listed in Section E. The neurotoxicological effects of of the selected chemicals are expected to occur gradually, not as a sudden overnight effect (inactivated proteins will require time to accumulate). However, if there is a suggestion of adverse effect near the end of a week, one of the investigators (or other TAD staff listed in Section E) will come in over the weekend. There will be ample time to detect changes during the week. As the neurotoxic changes occur, mild changes in gait and posture (not sufficient to interfere with walking, eating or drinking) are expected. At no point should the animals exhibit paralysis. We will be particularly observant for lethargy, piloerection, or prostration as signs of toxicity, and will be especially watchful for problems with mobility that create issues with eating or drinking.

Animals will be weighed daily prior to dosing and will be observed at that time. Additionally, even closer evaluations will be conducted during the weekly behavioral testing. If impaired mobility is observed, the animal will be monitored more frequently and the attending veterinarian will be consulted for appropriate treatment.

6. Non-surgical Category D or E procedures. Describe each procedure separately, include details on the following (Also fill in Section B.9).

a. Treatments (e.g. dosages, duration of exposure, route, volume, frequency):

Anesthesia: The animal is placed in the induction chamber located in the fume hood, and the lid closed and secured. Isoflurane gas level is set at 4-5% with 1-1.2 liters/minute flow rate. The animal remains in the induction chamber until breathing slows to approximately 2 seconds between breaths. After the gas flow is turned off, the lid is opened and the animal removed. To determine whether the animal is fully anesthetized, multiple firm toe pinches will be used. (If animal responds, it will be put back in induction chamber, the lid closed, and induction anesthesia resumed. If the animal does not respond, it is fully anesthetized.) The animal's thigh and ankle area are shaved using barber's trimmers. Then the animal is moved to the testing apparatus (in surgical suite), placed in a face mask and the isoflurane is turned on at an initial setting of 3-3.5% with a 1-1.2 liter/minute flow rate. A mild abrasive cream and alcohol wipe (as often used in humans

to improve electrode-skin conductivity) to clean the skin of dead cells and reduce tissue resistance will be used on the tail, shaved thigh area, and shaved ankle area. The mild abrasive will be removed with an alcohol wipe. No breaking of the skin will be produced. We will use (which are used in humans for this purpose) Lemon Prep and PDI Electrode Skin preparation pads (alcohol wipes with mild abrasive included). The percent isoflurane may be reduced during testing to 2.5-2%, while maintaining sufficient level of anesthesia that the animal is non-responsive to toe pinch. When testing is completed, anesthesia will be discontinued and the animal will be placed on a recirculating water heating pad until ambulatory (this takes only a few minutes) and returned to its home cage.

b. Blood Collection (Provide a description of the procedure including method, volume, and frequency if appropriate. Indicate if the procedure is survival or terminal. Include preparatory methods, descriptions of incisions, etc.):

N/A

c. Testing methods:

Nerve Excitability Testing

Animals will be tested under general isoflurane anesthesia to examine nerve excitability. The animal will be placed on the feedback controlled electric heating pad, with the rectal thermometer inserted at a depth of approximately 2.5 cm. When the animal is anesthetized and does not respond to multiple firm toe pinches, the isoflurane may be turned down to 2-2.5% during nerve excitability testing. Rectal temperature should be maintained at approximately 37 degrees Celsius.

Electrical stimuli will be applied via surface electrodes to the caudal motor nerve of the tail and tibial/sciatic nerve located above the ankle or at the hip, delivered using non-polarizable Ag/AgCl electrodes. The level of electrical stimulation varies between the tests in the nerve excitability procedures. It is the level of stimulus required to produce a criterion response that is one of the variables that is measured. It will vary between animals and tests within the nerve excitability testing procedures. Recording electrodes will be stainless steel needle electrodes. Specific configurations are described below. Upon recovery from anesthesia, no pain or distress is anticipated.

Nerve excitability testing will involve three components:

A. Recording tail compound muscle action potentials (CMAPs). To stimulate the caudal nerve, a cathode is placed at the base of the tail. The anode is attached to the left rear limb, in the inner thigh area. CMAPs from tail muscles are recorded. The active needle electrode is inserted posterior to the cathode and the reference needle electrode is distal to the cathode. The ground surface electrode (a disposable ring electrode) is placed between the cathode and active recording electrode on the tail.

B. Recording CMAPs from foot musculature. The cathode is placed on the left rear limb above the ankle and the anode is attached to the left rear limb, in the inner thigh areal. This configuration will stimulate the tibial nerve. The active needle electrode is placed in the plantar muscles in middle of the foot, and the reference needle electrode is placed at the base of the first digit. The ground surface electrode is placed in the same position as when stimulating the caudal nerve.

C. Recording sensory nerve action potentials. Two stimulating needle electrodes are located toward the tip of the tail, and two recording electrodes are located near the base of the tail. The needle electrodes are 27-gauge EEG electrodes. A ground electrode will be placed between the cathode and the CMAP recording electrodes.

SEP/CNAP/NCV testing:

Animals will be habituated to the restraint procedure for 2 days prior to testing to reduce distress (5 min on 1st day, 15 min on 2nd day). Immediately after each restraint session, the animal will be rewarded with a treat (e.g., sugar pellet, fruit crunchies, vanilla wafer). Approximately 7 days after surgery, the animals will be tested for SEPs/CNAPs/NCV. The animals will be restrained in a decapicone with their head, pinnae, and tail protruding. Masking tape will be placed over the rat's paws to prevent tearing of the decapicone. The front paws will be taped to each other and the hind paws will be taped to each other. Masking tape will be placed around the soft plastic cone near the rats head and tail region to prevent the rat from tearing or

backing out of the decapicone. The decapicone will then be placed in a custom built velcro strap assembly located inside a sound attenuated Faraday box. Different sized restrainers are available for different sized rats. The animals will be restrained for about 15 - 20 min. The animals' tails will be wiped with an isopropyl alcohol pad and placed in a Teflon tray with a semicircular tapered groove milled into the base and top portions. The tray contains 7 needle electrodes (25 gauge) which protrude in to the groove and penetrate the tail ~ 1.5 mm. Insertion of needle electrodes (syringe needles) will result in transient pain. The tail is taped to the base, a needle ground electrode is inserted in the dorsal tail, and the top portion of the tray is attached (which immobilizes the tail and holds the electrodes in place). Different sized trays are available for different sized rats. The needle electrodes are cleaned with 70% isopropyl alcohol between rats and replaced daily. Following testing, the animal is removed from the decapicone and the tape gently removed from the paws.

Recording somatosensory or peripheral nerve responses from rat appendages other than the tail generally requires limb restraint to prevent movement artifacts. Using the custom designed tail trays allows rats to be restrained using decapicones, while controlling tail movement. Thus, the procedure we use minimizes trauma to the rat. Brief biphasic (50 microsecond each polarity) constant current electrical pulses (3 mA maximum) are applied to the tail to elicit compound nerve action potentials. Testing three stimulation levels (1, 2, 3 mA) will require about 6 min of stimulation at 0.91 Hz (e.g. about 1 stimuli/sec). These low level electrical stimuli are not regarded as painful. Both central and peripheral nerve responses are recorded simultaneously from the same stimulus. These procedures have been used successfully in many previous studies.

The IACUC (including the AV), has viewed this procedure in person, including the restraint with tape and the electrodes that will be used in this testing LAPR. The procedure was categorized as Category E due to restraint. The IACUC (including the AV), watched the recovery period of the animal after removal from the restraint, which was observed to be short and smooth, and then animals are returned to home cages.

d. Restrictions placed on the animals' basic needs (e.g., food and/or water restriction, light cycles, temperature). Provide details regarding the length of restriction. Describe the method(s) for assessing the health and well-being of the animals during restriction. (Amount of food or fluid earned during testing and amount freely given must be recorded and assessed to assure proper nutrition.):

None

e. Describe how animals will be monitored (e.g., frequency of observations, by whom):

Nerve excitability testing: Animals will be monitored while under anesthesia with a pulse oximeter by Exemption 6 Exemption 6 Exemption 6

SEP/CNAP/NCV: The neurophysiological signal (EEG or CNAP) will be monitored on a computer screen during collection and the free running analog response on the oscilloscope for evidence of struggling or respiratory distress. These conditions will be evidenced by collection artifacts on the computer or large, low frequency, swings on the oscilloscope trace. Monitoring will be performed by Exemption 6 .

f. Analgesia (Category D Procedures) - list drugs, dosages, route of administration and frequency:

g. If treatment-related deaths are expected, this must be thoroughly justified. Death as an endpoint is highly discouraged:

No deaths are expected.

7. Surgical Category D and E procedures. Indicate if the surgery is survival or terminal. Describe each surgical procedure separately, include details on the following (Also fill in Section B.9)

a. Complete description of surgical procedure including presurgical preparation, aseptic technique, surgical closure, etc:

When survival surgery is performed the animals will be approximately 90-120 days old and are expected to be in the 500 - 600g weight range. Animals will not be treated with any test compound on the day of surgery.

For 2 days prior to surgery, the animals will be given a 5 g tablet "blank" to acclimate them to post-operative tablets containing Carprofen.

The surgeon will wear a face mask, sterile surgical gloves, and a clean laboratory coat. Surgical instruments are sterilized in a autoclave prior to surgery. Cortical electrodes will be sterilized either by autoclaving or by FDA-approved cold sterilization methods for devices using commercial solutions of hydrogen peroxide or a hydrogen peroxide peracetic acid solution. If cold sterilization is used, the electrodes will be rinsed with sterile saline, and stored in a sterile bag, before surgical procedures. Instruments will be hot bead sterilized (500 oF for 15s) between animals (maximum of 5 animals), and the stereotaxic instruments are wiped with 70% isopropyl alcohol. A sterile drape and the counter diaper are replaced between animals. Aseptic techniques for rodent surgery will be used for surgical procedures.

Anesthesia will be induced and maintained using isoflurane. Once the animal is anesthetized the incision location is shaved in a separate room, moved to the surgery location, and placed in a stereotaxic instrument. The cornea will be kept moist with artificial eye lubrication. Immediately prior to surgery, the animals will be administered Carprofen (5 mg/kg, sc). The incision location is cleaned with a 10% povidone-iodine solution followed by 70% isopropyl alcohol pads and new sterile surgical drape placed around the incision site. A pulse oximeter probe is attached to the rat's foot. Blood oxygen will be monitored to maintain a level of approximately 90%. An approximately 3 cm incision over the skull will be made, and the skull cleared of tissue and cleaned. Holes will be drilled through the skull at appropriate points for cortical screw electrodes (stainless steel, 00-903, 1/16" soldered to Nichrome wire).

The electrodes will then be implanted in the skull. Cortical electrodes will consist of stainless steel screws. The electrodes are connected to a head cap which is then attached to the skull using cranioplastic powder and cement. The wound is cleaned with saline solution, closed with surgical staples, and neomycin and polymyxin B sulfates, and bacitracin zinc ophthalmic ointment applied around the incision. 100% oxygen may be provided during the last 1-2 min of the procedure to speed awakening. Animals may be injected subcutaneously with USP 0.9% saline at about 10-15 ml/kg. Impedance of the electrodes at surgery is recorded. The animals will be placed on a recirculating water heating pad (37° C) until awakening. They will then be returned to their home cages, and a few food pellets placed in the bedding. 24 and 48 h post surgery, the animals will be given a tablet containing 2 mg Carprofen as a supplemental analgesic. As recommended by the Attending Veterinarian, we will consider adding pain scoring to this LAPR at a future date.

b. Anesthetic regimen (Drugs, dosages, volume, route of administration and delivery schedule). The use of paralytic or neuromuscular blocking agents w/o anesthesia is prohibited:

Rats will be placed in an induction chamber (maximum of 2 at a time in dual compartment induction chamber) located in the fume hood with a secured lid. Isoflurane gas at 4-5% with 1-1.2 liters/minute flow rate will be used for induction. Animals will remain in induction chamber until breathing slows to approximately 2 seconds between breaths. The gas and flow rate will then be stopped and the animal removed. The head will be shaved, and the animal placed in the stereotaxic device. The rat's nose will be placed in the surgical nose cone and anesthesia maintained with 3-3.5% isoflurane (with a verified 1-1.2 liter/minute flow rate). When the animal reaches surgical level of anesthesia (as evidenced by lack of response to a firm toe pinch), the isoflurane may be reduced to 2-2.5%. A pulse oximeter is clipped onto the animal's foot. The pulse oximeter should read above 90% oxygen saturation. Optimal O2 saturation is > 95%. If O2 saturation drops below 90%, the anesthesia should be turned down at 0.5-1.0% intervals, and the oxygen flow rate increased slightly until the oxygen saturation increases to above 90%. Multiple spare O2 tanks are maintained in the laboratory to assure adequate supply. Surgery will be initiated as described in Section 7a.

c. Postoperative care (thermal support, special feeding, responsible personnel, removal of sutures/staples, frequency and duration of monitoring including weekend and holiday care):

Rats will be placed on a recirculating water heating pad (37°C) until awakening. They will then be returned to their home cages and food will be placed on the bedding for easy access. A Carprofen (2 mg) tablet will be placed in the cage 24 and 48 h post surgery as an analgesic. Animals will be allowed approximately 7 days recovery time before testing. **Exemption 6** are responsible for daily monitoring of the animals during recovery. If infection occurs post surgery, the Attending Veterinarian will be consulted.

Surgical staples are not removed, as even the brief anesthesia required for this procedure will compromise experimental results. Animals will be given approximately 1 week to recover prior to testing. Usually, the

animals are tested and euthanized within 2 weeks of surgery. Any animals remaining on study longer than 2 weeks will be restrained by hand and any remaining wound clips will be removed.

d. Post operative analgesics (drugs, dosage, and volume and route of administration, frequency):

48 and 24 h prior to surgery animals are given a 5 gram blank (no analgesics) tablet to acclimate them to post-operative tablets. One Carprofen tablet (2 mg) will be given to the animals 24 and 48 h after surgery

e. Will any animal be subject to more than one surgical procedure over the course of its lifetime, either here at NHEERL or elsewhere?

○ Yes ● No

f. Identify any surgical procedures performed at other institutions or by vendors:

N/A

8. Humane interventions (for treatments/procedures in all categories).

a. What resultant effects, if any, do the investigators expect to see following procedures or treatment? Please include transitory as well as permanent effects. Examples might include lethargy, ataxia, salivation or tremors. Indicate the expected duration of these effects.

Chemical dosing: Based on the limited literature available, no adverse effects have been reported by other investigators. However, potential neurotoxic effects with repeated exposures have not been investigated. If the predicted neurotoxic changes occur, mild changes in gait and posture (not sufficient to interfere with walking, eating or drinking) are expected. At no point should the animals exhibit paralysis. We will be especially watchful for problems with mobility that create issues with eating or drinking. If such problems are observed, the animal will be humanely euthanized. Changes in health status will result in a health report and notification of the attending veterinarian.

Nerve excitability testing: Animals will be tested and monitored while under anesthesia. Level of isoflurane will be adjusted to maintain surgical levels of anesthesia (as indicated by lack of response to multiple firm toe pinches).

SEP/CNAP/NCV: If oscilloscope traces indicate signs of struggling or respiratory issues, the experiment may be paused and the animal examined for suitability to continue (e.g. stops struggling, fix any issues with restraint or electrodes). If there appears to be respiratory distress or excessive struggling, the animal will be returned to its home cage.

b. State the criteria for determining temporary or permanent removal of animals from the study. Describe actions to be taken in the event of deleterious effects from procedures or chemical exposures. Describe actions to be taken in the event of clinical health problems not caused by procedures or exposures.

Animals may be temporarily or permanently removed from the study due to signs of non-transient pain, distress, or hindlimb paralysis. Signs of pain and distress will include lethargy and decreased muscle tone, mild tremors, raspy breathing, or repeated and prolonged vocalizations not associated with handling and testing. Overall changes in health status such as a rough, ungroomed coat, or weight loss (as opposed to less weight gain) will also be monitored. We will be especially watchful for problems with mobility that create issues with eating or drinking. If such problems are observed, the animal will be humanely euthanized. If the attending veterinarian diagnoses an animal as in unrelievable pain or untreatable distress, the animal will be removed from the study and humanely euthanized

If a post-surgical infection occurs, the attending veterinarian will be consulted. Historically, this has been rare, and the treatment involves applying additional topical antibiotic. During testing, the EEG will be monitored on oscilloscopes, and evoked responses observed on the computer terminal. Sustained large changes in the EEG will be evidence of struggling, and the experiment will be paused and the animal examined for suitability to continue. If there appears to be respiratory distress or excessive struggling, the animals will be returned to their home cage. In the infrequent case of a malfunction or removal of headcap, the animal will be permanently removed from the study and humanely euthanized.

9. Alternatives to pain and distress (Category D and E Procedures only). Provide narrative regarding the sources consulted to ascertain whether acceptable alternatives exist for potentially painful/distressful procedures. Include databases searched or other sources consulted, the date of the search and years covered by the search, and key words and/or search strategy used. Assistance with searches is available

through the EPA Library Staff.

Nerve excitability testing occurs under anesthesia. No pain or distress should result.

SEP/CNAP/NCV testing. Our historical database of SEP/CNAP/NCV testing (tail nerves) has occurred in non-anesthetized animals, commonly as part of a multisensory test strategy (which included cortical responses to somatosensory or visual stimuli). Cortical responses (produced by peripheral nerve stimulation) are dramatically altered by anesthesia, limiting their application to detect subtle environmental toxicant-induced changes in anesthetized preparations. As a general mechanism of action, anesthetics affect nerve function, and may also have effects on peripheral nerves.

The custom designed tail trays allows rats to be restrained using decapicones, while controlling tail movement. Using surface electrodes for stimulation and recording requires removing the scales from the rat tail to allow adequate electrical contact; this procedure is more traumatic than using needles to penetrate the scales. Thus, the procedure we use minimizes trauma to the rat.

The brain's responses vary over the different regions of the cortex. Thus, to maintain constant recording locations between animals (thus reducing experimental variance and the number of animals required) stereotaxic surgery will be used. This implantation will assure that the electrodes will not move during the testing procedure, which must be performed in awake animals. Evoked potential testing involving cortical responses should be performed in non-anesthestized subjects due to the known impact of anesthetics on cortical responsiveness.

Recording somatosensory or peripheral nerve responses from any rat appendage other than the tail requires either anesthesia or severe restraint to prevent movement artifacts. Using the custom designed tail trays allows rats to be restrained using decapicones, while controlling tail movement. Using surface electrodes for stimulation requires removing the scales from the rat tail to allow adequate electrical contact. This procedure is more traumatic than using needles to penetrate the scales. Our testing procedure allows simultaneous recording of peripheral nerve, cortical somatosensory, and cerebellar somatosensory responses from a single stimulus. Thus, the procedure we use minimizes trauma to the rat.

Since the evoked responses are recorded from functioning brain tissue, there are currently no viable in vitro alternatives to predict in vivo changes in cortical sensory function. Therefore replacement of an in vivo preparation in not possible at this time.

Literature Searches:
1. Citronellal - A PubMed Search using terms "citronellal and neurotoxicity" (no quotes in search; no year limits) resulted in no results. A search using terms "citronellal and nerve function" resulted in 3 hits. The first is irrelevant, the second is an in vitro frog preparation, and the third involved inhalational exposure - no effects reported. .

2. Phenylacetaldehyde - A PubMed Search using terms "phenylacetaledhyde and neurotoxicity" (no quotes in search; no year limits) resulted in 1 result which is not relevant. A search using terms "phenylacetaledhyde and nerve function" resulted in 3 hits, none of which are relevant.

3. CNAPs: A Google search using terms "alternatives to needles for unanesthetized animals in cnaps" (no quotes in search; 31 hits) returned no relevant results (see attached). PubMed Search using terms "compound nerve action potential in rat caudal tail nerve" (no quotes in search) resulted in eight results. The first two are nerve excitability papers. The third is from our laboratory, using our standard non-anesthetized models. The fourth is a disease model that creates severe changes in nerve function that were detectable in an anesthetized preparation. The fifth and eighth results use environmental chemicals or mild pharmacological manipulations in non-anesthetized rodents. The sixth and seventh results used anesthetized rats with a severe nerve compression model which produced almost complete nerve block. The eighth result used non-anesthetized rats, very similar to our preparation. Google Scholar Search using terms "alternatives needles non-anesthetized cnap" (no quotes in search; 15 hits, only 1 relevant hit which is from our laboratory).

4. SEPs: A PubMed search was performed (no year restrictions), using keywords: cortical visual neurophysiology and rats and anesthesia returned 1 non-relevant result. A PubMed search was performed (no year restrictions), using keywords: cortical somatosensory neurophysiology and rats and anesthesia. Hits related to somatosensory responses indicate changes in evoked responses produced by anesthesia

(see attachments). Therefore, to maintain biological relevance to toxicant-induced changes, a non-anesthetized preparation is required.

SECTION C - Animal requirements

Describe the following animal requirements :

1. Indicate the number of animals required over the study period for this protocol. Please enter numbers only.

a. Animals to be purchased from a Vendor for this study: 200

b. Animals to be transferred from another LAPR:

LAPR Number that is the source of this transfer:

c. Animals to be transferred from another source:

d. Offspring produced onsite (used for data collection and/or weaned):

e. TOTAL NUMBER of animals for duration of the LAPR 200

2. Species (limited to one per LAPR): Rat(s)

3. Strain: Long Evans Rat(s)

Describe special requirements for animals with altered physiological responses (e.g., genetically altered, aged)

N/A

4. Sources of animals:

Charles River; About 2 months old at study initiation; about 250 g

5. Provide room numbers where various procedures will be performed on animals:

Exemption 6Exemption 6

6. Will any animals be housed in areas other than the animal facility longer than 12 hours? If so, state location. Such areas require prior IACUC approval as a satellite facility before LAPR can be reviewed.

No ***Room Numbers:***

7. Describe any transportation and containment methods involved in moving animals between EPA buildings, or between EPA and other institutions (excluding any commercial shipments)

Animals will be moved from the animal colony to laboratory space Exemption 6 using housing racks for testing. Cages will be covered with a filter top, and cages and cart wheels will be sanitized before returning to animal facility.

8. Describe any unusual housing or husbandry requirements, or acclimation requirements. Justify any treatment beginning less than 3 days after arrival.

None

9. Describe special assistance requested of the animal contract staff, including procedures and dosing. NOTE, this request must be submitted separately to the Animal Resources Program Office (ARPO)

None

10. Housing and Enrichment.

The IACUC encourages the use of environmental enrichment whenever possible (see IACUC website for details). Provide details on how the animals will be housed, including type of cage

(e.g., solid bottom or wire screen), bedding material, number of animals per cage, and environmental enrichment. Note that housing rodents individually without environmental enrichment requires justification.

We will attempt to pair house the animals during the Step 1 studies. During the Step 2 studies, we have concerns about having an even number of each treatment group in each squad. Co-housing different treatment groups may be problematic because the controls will potentially be exposed to the test chemical or metabolites via the cage mates' exhaled breath or excreta. Also, behavioral effects in treated animals could affect the social dynamics and social hierarchy, introducing a confounder. We will pay close attention to the animals during the Step 1 studies for such interactions, try to pair animals from the same dose group together.

When animals are surgically implanted, we will change to single housing to avoid post-surgical complications from aggression between males. Animals will be singly housed in polycarbonate cages with heat treated pine shaving bedding and EnvroDri for enrichment.

SECTION D - Agents Administered to Animals

1. Identify all hazardous and non-hazardous agents to be administered to living animals. For agents requiring a Health and Safety Research Protocol (HSRP), provide the title of the approved HSRP for each such agent. If no protocol is required for an agent deemed potentially hazardous (e.g. nanoparticles, recombinant DNA), describe the safety precautions to be used.
Provide maximum dosing levels and route-appropriate LD50s (where available) for each agent used for dosing.

Isoflurane (pharmaceutical grade): Isoflurane is an inhalation anesthetic and is considered a "potentially hazardous substance" but does not require a HSRP. Rats will not be exposed to more than a 5% concentration of a brief (5 min) period of time. Rat oral LD50 = 4,770 mg/kg. Rat inhalation LC50 = 15,300 ppm (1.53%) for 3 h.

LemonPrep Skin Prep Lotion - LD50 not available.
PDI Electrode Prep Pads - rat LD50 4,396 mg/kg based on rubbing alcohol (isopropanol) in pad.

Carprofen (LD50 oral = 149 mg/kg; sc not available) will be injected (sc; 5 mg/kg) prior to surgery, and tablets containing 2 mg Carprofen will be provided for 2 days post surgery.

Povidone-Iodine 10% USP grade. Topical antibiotic. Rat oral LD50 = 8,000 mg/kg.

Neomycin and Polymyxin B Sulfates, and Bacitracin Zinc Ophthalmic Ointment (pharmaceutical grade) is a topical antibiotic recommended by the attending veterinarian. Mineral Oil (component of antibiotic creams) LD50 Rat oral rat >= 5,000 mg/kg. Polymyxin B LD50 oral = 500 mg/kg.

100% Corn oil, Food grade. LD50 Rat Oral > 90 g/kg

Citronellal (LD50 Rat oral = 2420 mg/kg). We will start with estimated LD50 * 0.1 = 242 mg/kg/day, and adjust concentrations based on what is observed in the animals. We anticipate that this will be the maximal dose. We cannot find any 30-60 day oral toxicity studies for citronellal.

Phenylacetaldehyde (LD50 Rat oral = 1,550 mg/kg). We will start with LD50 * 0.1 = 155 mg/kg/day, and adjust concentrations based on what is observed in the animals. We anticipate that this will be the maximal dose. We cannot find any 30-60 day oral toxicity studies for phenylacetaledhyde.

2. Describe compounds to be administered to animals.
a. Are all substances pharmaceutical grade? If not, provide a scientific justification for the use of non pharmaceutical grade compounds.
No. Citronellal and Phenylacetaldehyde are not available in pharmaceutical grade.

b. Describe any plans to administer human or animal tissues, blood or body fluids to the animals in the LAPR. Provide information to assure that such material is pathogen free. Indicate what safety precautions are necessary for handling the material.

N/A

c. Provide a statement regarding any safety precautions necessary for handling any of these materials.

N/A

NOTE: Any unresolved health/safety questions which arise during IACUC review of this LAPR will require consultation with the Safety, Health, and Environmental Management Office.

SECTION E - Personnel Training and Experience

1. Identify all project personnel conducting animal experimentation. Specify the techniques for which they have responsibility, and their relevant training and experience. Additional personnel may be added to the table below as a group (by Division) for Category C procedures. By so doing you are giving assurance that these personnel have received all required training and are qualified to perform the Category C techniques requested.

Use this area to type in additional personnel information not available in the table drop-down lists:

Hint: *The names in the first 2 lines of the table below are filled automatically from the Principal Investigator & Alternate Contact fields. A new line will be made available when a name is selected & upon leaving the name field (i.e. tabbing or clicking in another field).*

NAME	ROLE	SPECIFIC RESPONSIBILITY	RELEVANT TRAINING
Exemption 6	Principal Investigator	Experimental design, dosing, surgery, animal testing, data analysis	> 30 years toxicology testing experience. All required IACUC training up-to-date. Experience with surgical, behavioral, and neurophysiological testing.
Exemption 6	Technical Staff	Experimental design, dosing, surgery, animal testing, data analysis	About 1 year physiological testing experience. All required IACUC training up-to-date. Experience with surgical and neurophysiological testing.
Exemption 6	Technical Staff	Dosing, behavioral testing, and data analysis	> 30 years behavioral testing experience. All required IACUC training up-to-date.
Exemption 6	Technical Staff	Dosing, surgery, behavioral and neurophysiological testing and data analysis	About 20 years neurotoxicological testing experience. All required IACUC training up-to-date. Experience with behavioral and physiological techniques and surgical procedures.
Exemption 6	Student	Assist with surgery, testing, and data analysis	All required IACUC training up-to-date. Will work under supervision of Exemption 6 and/or Exemption 6
Exemption 6	Technical Staff	Dosing	About 20 years neurotoxicological testing experience. All required IACUC training up-to-date. Experience with dosing and behavioral techniques.

RTP-NHEERL	Tech Support	Category C Procedures	All NHEERL required training is complete.

SECTION F - Animal Breeding Colonies

This section pertains to the breeding of animals for maintenance of ongoing animal colonies. Do not include breeding that is part of experimentation and accountable under Section C.

Describe:

1. Estimated number of breeding pairs and liveborn per year N/A

2. Breeding protocols and recordkeeping N/A

3. Methods for monitoring genetic stability N/A

4. Disposition of all offspring and retired breeders that are not used in accordance with the procedures described in this LAPR N/A

SECTION G - Euthanasia

1. When will the animals be euthanized relative to experimental procedures?

Request for euthanasia will be submitted to Animal Care Staff within 1 week after completion of testing procedures. Animals not used in the study will be euthanized within 1 week of conclusion of the study by the animal care staff upon request.

2. Describe the euthanasia techniques:

Method(s): CO2 asphyxiation or as determined by Animal Care Staff
Agent(s): CO2
Dose (mg/kg): to effect
Volume:
Route: inhalation

Source(s) of information used to select the above agents/methods:

Veterinary Staff, 2013 AVMA Guidelines on Euthanasia.

3. Provide justification and references for any euthanasia agent or method that is not consistent with recommendations of the American Veterinary Medical Association (AVMA) Guidelines for Euthanasia (e.g., cervical dislocation or decapitation without anesthesia; cervical dislocation in rodents weighing more than 200 grams).

4. Describe how death is to be confirmed.

Prolonged absence of breathing

SECTION H - Disposition of Used and Unused Animals

Describe the disposition of any animals remaining after project completion.

Euthanized by Animal Care Contractor
Transferred to another study

The IACUC encourages investigators to reduce the overall number of animals used at NHEERL. Would you consider transferring any unused animals from this LAPR to another approved LAPR?

● Yes ○ No

SECTION I - Assurances

1. Animals will not be used in any manner beyond that described in this application without first obtaining formal approval of the IACUC.
2. All individuals involved in this project have access to this application, are aware of all EPA policies on animal care and use, and are appropriately trained and qualified to perform the techniques described.
3. Thorough consideration of the three "R"'s (Replacement, Reduction, Refinement) has been given, as applicable, to a. the use of animals, and b. procedures causing pain or distress (with or without analgesia/anesthesia), including death as an endpoint. The minimum number of animals required to obtain valid experimental results will be used.
4. The Attending Veterinarian has been consulted in regard to any planned experimentation involving pain or distress to animals.
5. The IACUC and Attending Veterinarian will be promptly notified of any unexpected study results that impact the animals' well-being, including morbidity, mortality and any occurrences of clinical symptoms which may cause pain or indicate distress.
6. All procedures involving hazardous agents will be conducted in accordance with practices approved by the Safety, Health, and Environmental Management Office.
7. I certify that I am familiar with and will comply with all pertinent institutional, state and federal rules and policies.
8. The IACUC has oversight responsibilities for animal care and use, and may request consultation or feedback regarding the conduct of in vivo procedures, progress and accomplishments, and any problems encountered.

EPA Principal Investigator	Certification Signature Date
Exemption 6 Exemption 6	11/02/2016

Submitted: 11/04/2016

Certification:

Certification by EPA Supervisor (Branch Chief or Division Director) that the project described herein has been reviewed and approved on the basis of scientific merit:

Branch Chief/Division Director	*Approval Date*	*Phone Number*	*Division*	*Mail Drop*
Exemption 6	11/09/2016 by Exemption 6 Exemption 6 Exemption 6 Exemption 6 RTP/USEPA/US	Exemption 6 *Lotus Notes Address* Exemption 6 Exemption 6 Exemption 6 Exemption 6 Exemption 6 /RTP/USEPA/US	TAD *Branch* DTB	MD *Submitted to Branch Chief for Approval* 11/04/2016 05:23 PM

ATTACHMENTS

19-11-003 PI resp2.pdf 19-11-003 PI resp.pdf New Exemption 6 Exemption 6 11-16.pdf Phenylacetaldehyde_Neurotoxicity_PubMed.pdf
Pphenylacetaldehyde_nerve_function_ PubMed.pdf Citronellal_Neurotoxicity_PubMed.pdf
Citronellal_nerve_ function_PubMed.pdf ((cortical somatosensory neurophysiolog...pdf

Migraine photophobia originating in con...iven retinal pathways.pdf

alternatives needles non-anesthetized cnap - Google Scholar.pdf

compound nerve action potential in rat caudal tail nerve - PubMed - NCBI.pdf

alternatives to needles for unanesthetized animals in cnaps - Google Search.pdf

Actions

First Update notification sent: 12/04/2017
Second Update notification sent:
First 2nd Annual notification sent:

Second 2nd Annual notification sent:

1st Expiration notification sent:
2nd Expiration notification sent:

History Log: